DEVELOPMENTAL BIOLOGY **188,** 322–336 (1997)
ARTICLE NO. DB978655

# of Neuronal PC12 Cells and Developing Sympathetic Neurons *in Vivo*

Songli Wang,*,[1] Angela J. DiBenedetto,†,[1] and Randall N. Pittman*

**Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; and †Department of Biology, Villanova University, Villanova, Pennsylvania 19085*

To identify primary response genes induced during early stages of neuronal programmed cell death (PCD), we screened by differential hybridization a subtracted cDNA library prepared from neuronal PC12 cells deprived of NGF for 6 hr in the presence of cycloheximide. Eight induced cDNA sequences were identified and designated *message up-regulated during death (mud)-1–8.* To determine which cloned sequences might be involved in neuronal PCD *in vivo,* expression of *mud* genes was analyzed in developing rat superior cervical ganglia (SCG) undergoing programmed cell death, using a combination of reverse Southern, reverse transcription polymerase chain reaction (RT-PCR), and *in situ* hybridization. Five sequences (*mud-1, -3, -5/8, -6,* and *-7*) are induced in SCG undergoing cell death *in vivo,* and induction of at least three of these (*mud-3, -6, and -7*) occurs in neurons. Partial sequence analysis reveals that *mud-1* corresponds to *annexin VI; mud-3* corresponds to rat *PC3,* mouse *TIS21; mud-4* appears to be the rat homolog of human *TAFII70; mud-5* and *-8* are >85% identical members of the rodent gene family of B2-transcribed repeats; and *mud-6* appears to be the rat homolog of human *Ring 3* and Drosophila *female sterile homeotic* (*fsh*). *Mud-2* and *mud-7* encode novel sequences. These new candidate genes provide markers for early stages of neuronal PCD, are potentially involved in the cell death process, and serve to expand our view of cell death control in the developing nervous system. © 1997 Academic Press

INTRODUCTION

Physiological cell death has emerged as a fundamental biological process essential to the maintenance of proper cell number and the defense against viral invasion, oncogenic transformation, and other forms of cellular damage (reviewed in Hale *et al.,* 1996). It was first recognized, however, as an important and ''programmed'' event during development in studies of vertebrate embryology and insect and amphibian metamorphosis (Hamburger and Levi-Montalcini, 1949; Saunders, 1966; Tata, 1966; Lockshin, 1981). Programmed cell death (PCD) during normal development serves many different functions, including the deletion of cell populations that are unwanted or no longer needed and the formation of sex-specific structures (for reviews: Oppenheim, 1991; Schwartz, 1992; Naruse and Keino, 1995; Sanders and Wride, 1995). Since the final purpose of PCD depends on the cell populations involved and the time of its occurrence, careful analysis of the process in each particular instance is needed for an in depth understanding of the normal biology of programmed cell death.

Cell death is particularly striking as part of the normal developmental program governing the formation of the vertebrate nervous system. Maturation of the nervous system requires that nearly half of all neurons die during the period when neurons are making functional connections with their targets (reviewed in Oppenheim, 1991). Neurons at this time appear to compete for limited amounts of, or limited access to, target-derived trophic factors, and to die by apoptosis if they fail to obtain a sufficient supply (reviewed in Korsching, 1993). Neuronal PCD during development may thus provide a *de facto* mechanism for the proper matching of neuron number and target size. An example of this programmed cell death occurs in rat superior cervical ganglia (SCG) in a 3-day perinatal period during which nearly 35% of the neurons die (Wright *et al.,* 1983).

Although PCD is often initiated by signals from the extracellular environment, its execution is mostly cell autonomous. Studies using macromolecular synthesis inhibitors *in vivo* and *in vitro* have demostrated the dependence in many instances of PCD on RNA and protein synthesis

[1] Equal contribution by first two authors.

0012-1606/97 $25.00

(Tata, 1966; Martin *et al.,* 1988; Oppenheim *et al.,* 1990; Schwartz *et al.,* 1990; Scott and Davies, 1990) and have led to the notion that a cascade of gene expression and the accumulation of ''killer'' proteins may be required to effect cell death (Martin *et al.,* 1988). In both *C. elegans* and Drosophila, true ''killer'' proteins have been identified (Yuan and Horvitz, 1990; White *et al.,* 1994; Steller, 1995); similarly, proteases of the *ced-3*/ICE class appear to be involved in many cases of mammalian PCD (Patel *et al.,* 1996; Orth *et al.,* 1996). These functions may provide a final common mechanism for cell death. In contrast, the regulatory signals that govern the death decision are likely to be specific to cell type and developmental context. Indeed, like other pathways that determine a cell's response to a relevant signal, the cell death program in mammals appears to consist of complex patterns of gene activation and repression that identify regulatory molecules linking the initial stimulus to die with the synthesis or activation of actual killer functions (Estus *et al.,* 1994; Mesner *et al.,* 1995; Park *et al.,* 1996). The elucidation of both universal and cell-type specific mechanisms of cell death control will be crucial to our understanding of the process in development.

Changes in expression of specific genes have been associated with neuronal PCD *in vivo,* and include: *c-jun* and *c-fos* induction in dying neurons after axotomy or ischemia; Rb deficiency in knockout mice resulting in the excess developmental death of neurons in brain and spinal ganglia; *c-rel* induction during cell death in developing avian embryo; and induction of a gene of the CAD family of transcriptional activators in PCD of skeletal muscle in Maduca sexta (Jacks *et al.,* 1992; Lee *et al.,* 1992; Abbadie *et al.,* 1993; Dragunow *et al.,* 1993; Smeyne *et al.,* 1993; Symonds *et al.,* 1994; Sun *et al.,* 1996). Recently, entire patterns of gene expression have been identified using *in vitro* paradigms of trophic factor deprivation-induced neuronal cell death such as NGF-dependent primary cultures of sympathetic neurons and neuronal PC12 cells (Freeman *et al.,* 1994; Estus *et al.,* 1994; Ham *et al.,* 1995; Mesner *et al.,* 1995). In these studies, candidate genes screened for induction were preselected on the basis of their involvement in other instances of death (reviewed in Freeman *et al.,* 1993), or because they represent a ''suspect'' class of genes, such as immediate early response genes (IEGs) or cell cycle regulatory genes, that has been implicated in the control of both apoptosis and mitosis (Ucker, 1991; Rubin *et al.,* 1993; Meinkrantz and Schlegel, 1995). This approach has been quite successful and has provided the means to monitor the intracellular signals and events active during the cell death process. For example, these studies have strengthened the association between cell death and cell cycle control and are consistent with the idea that proliferative stimuli can be lethal in neurons. In addition, the products of *c-jun* and some *fos* family members have been identified as necessary for cell death in an *in vitro* model of neuronal PCD triggered by loss of trophic support (Estus *et al.,* 1994; Ham *et al.,* 1995). The approach of examining preselected genes is limited, however, by the present conceptual framework that defines what a candidate gene might be.

In this study, we have used a neuronal PC12 cell system (Pittman *et al.,* 1993) and a subtractive hybridization strategy to clone new candidate genes for involvement in neuronal PCD. Using this approach, two entirely novel and five newly death-associated genes were identified. Among these are genes implicated in the control of global transcription, chromatin structure, splicing, and/or RNA polymerase III transcription. Most importantly, we identify five sequences induced in rat sympathetic ganglia (SCG) undergoing programmed cell death *in vivo,* and we demonstrate by *in situ* hybridization that induction of at least three genes occurs in neurons. The results implicate the cloned genes as potential players in developmentally programmed death of neurons in the intact animal, validate neuronal PC12 cells as a model for PCD *in vivo,* and expand our concept of apoptosis control.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise.

Cell Culture

PC12 cells were grown and differentiated as previously described (Pittman *et al.,* 1993; Mills *et al.,* 1995), on type I collagen in RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 5% fetal calf serum and 10% horse serum (Hyclone Laboratories, Logan, UT) for 7–8 days in the presence of 100 ng/ml 2.5S NGF (Collaborative Research, Bedford, MA). Neuronal PC12 cells were then subcultured onto collagen-coated plates in DMEM/F12 (1:1) medium (JRH) containing 5% fetal calf serum, supplemented with insulin (5 μg/ml), transferrin (10 μg/ml), selenium (30 μM), progesterone (20 nM), putriscine (100 μM), BSA (100 μg/ml), and 2.5S NGF (100 ng/ml), and maintained for another 4–5 days. NGF was removed from the cultures by adding NGF-free medium containing 50 μg/ml anti-NGF IgG. For some experiments, 25 μM neurotoxic amyloid peptide Aβ 25-25 was added to neuronal PC12 cells cultured in NGF.

RNA Preparation

For library construction and Northern blot analysis. Neuronal PC12 cells were incubated either in NGF-containing medium (control cells) or NGF-free medium containing anti-NGF IgG and 10 μg/ml cycloheximide (programmed cells) for 6 hr. Total RNA (1–2 mg from 10–12 15-cm dishes of PC12 cells) for control and programmed library construction was prepared using an acid guanidinium/phenol/chloroform protocol (Chomoczynski and Sacchi, 1987). Poly (A)+ mRNA (typically 10–12 μg) was isolated using the Fast Track kit (Invitrogen, San Diego, CA).

From superior cervical ganglia (SCG). One-day-old rat pups were injected subcutaneously with either saline solution or anti-NGF antibodies and SCG were dissected at designated times after injection. Poly(A)+ RNA was isolated directly from ganglia using MicroFastTrack (Invitrogen) and coprecipitated with glycogen as per manufacturer's protocol.

Construction of Source and Subtracted Libraries

Source libraries. Two directionally cloned source libraries were constructed using mRNA from programmed and control cell populations, respectively. Five micrograms of poly(A)+ RNA was converted into double-stranded cDNA using a XhoI-oligodT adaptor primer and the ZAP-cDNA Synthesis kit (Stratagene, La Jolla, CA). After ligation with *Eco*RI linkers and digestion with *Xho*I and *Eco*RI, cDNA inserts were ligated to *Eco*RI/*Xho*I-digested Lambda ZAPII as recommended, and the ligation was packaged into phage particles using the Gigapak Plus-6 packaging system (Stratagene). A total of $>10^6$ independent recombinants, determined by blue/white selection, was obtained for each source library.

Subtracted library. Single-stranded phagemid DNA (representing antisense cDNA) from the programmed library and sense RNA from the control library were used in subtractive hybridization of common sequences for the construction of an enriched programmed library. Whole library excision of single-stranded phagmid DNA was done according to the Stratagene protocol, using lambda phage from the programmed library and a fivefold excess of ExAssist helper phage. For *in vitro* transcription of sense RNA driver: Purified lambda DNA (15–20 μg) from the control library was linearized with *Xho*I and used as a template in large-scale (200 μl) transcription reactions as in Owens *et al.* (1991) with 50U T3 polymerase (Stratgene), 50–100 μg of sense RNA was labeled with photobiotin according to the method of Welcher *et al.* (1986).

Subtractive hybridization protocols were based on Owens *et al.* (1991): Biotinylated sense RNA (150 μg) and ssDNA preparation (7 μg) was coprecipitated and dissolved in 2× Pipes hybridization buffer {0.1 *M* PIPES [piperazine-N m N′-bis(2-ethanesulfonic acid)], pH 6.8, 1.2 *M* NaCl, 2 m*M* EDTA, 0.2% SDS}, and an equal volume of deionized formamide was added. Samples were heated at 95°C for 1 min and then incubated at 42°C for 24 hr. To separate subtracted ssDNA from hybridized sequences, reactions were diluted 10-fold with strepavidin binding buffer (10 m*M* Tris–HCl, pH 7.4, 400 m*M* NaCl, 2 m*M* EDTA) and incubated with streptavidin for 30 min at 60°C. The samples were then extracted with phenol/chloroform, incubated 60 min at 65°C in 0.2 *N* NaOH, and subjected to a second round of subtraction. Subtracted ssDNA (2–5 ng) from the programmed library was made double-stranded by one round of PCR amplification with 25–30 pmol of T3 polymerase primer (Stratagene) and 2.5U Taq polymerase (Perkin–Elmer, Branchburg, NJ), using standard conditions (Perkin–Elmer). *Escherichia coli* was directly transformed with this repaired plasmid preparation and spread on IPTG/Xgal-containing plates to create the subtracted library.

Subtractive Hybridization Screening

Synthesis of enriched cDNA probe. cDNA was prepared from 5–10 μg of poly(A)+ RNA from either programmed or control cells using 5–10 μg of oligo dT and 400U of MLV reverse transcriptase (Pharmacia, Piscataway, NJ), as in Sambrook *et al.* (1989). Reactions were incubated at 37°C for 2 hr and treated with NaOH to hydrolyze the RNA. Unincorporated nucleotides were removed by chromatography on BioGel P-60 (Bio-Rad, Rockville Cntr, NY).

Before radiolabeling, cDNA from programmed cells was subtracted twice by hybridization with a 20-fold excess of biotynylated sense RNA synthesized from the control library as described above. Unhybridized enriched cDNA (75 ng) was radiolabeled to high specific activity using random oligonucleotide primers, α-[^{32}P]-dCTP (3000 Ci/mmol; NEN Dupont, Wilmington, DE) and Klenow (Life Technologies, Grand Island, NY) according to Sambrook *et al.* (1989). This enriched probe was used for selective screening as described in the Results. Unenriched probes for conventional differential hybridization screening were prepared from cDNA radiolabeled to high specific activity as above, without prior subtraction.

Screening colonies. White colonies (2300) from the subtracted library were transferred to duplicate master plates and screened by colony blot hybridization at 42°C for 48 hr in 50% formamide, 4× SSPE, 0.1% SDS, salmon sperm, and 5× Denhardt's solution (Amersham, Arlington Heights, IL). Duplicate nitrocellulose filters (Schleicher and Schuell, Keene, NH) were hybridized with $1–3 \times 10^6$ cpm/ml of either enriched cDNA probe from programmed cells, or unenriched cDNA probe derived from control cells, and colonies giving positive signals with the enriched probe but not the control probe were selected for further rounds of screening.

Nucleic Acid Blotting

Northern blots. 5 μg of poly(A)+ RNA prepared from control and programmed neuronal PC12 cells was fractionated on formaldehyde agarose gels, transferred to GeneScreen filters (NEN Dupont) in 10× SSPE, and hybridized with $1–2 \times 10^6$ cpm/ml of radiolabeled gel-purified cDNA inserts in 7% SDS, 0.4 *M* phosphate buffer, pH 7.2, 1% BSA, and 20 m*M* EDTA at 60°C for 16–24 hr.

Reverse Southern blots. cDNA inserts from positive clones were amplified with flanking M13 foward and reverse vector-derived primer sequences and Taq polymerase (Perkin–Elmer), using manufacturer's PCR reaction conditions. Equal amounts of amplified product from each cloned insert were subjected to electrophoresis in duplicate agarose gels and were blotted onto GeneScreen filters (NEN Dupont). Duplicate filters were hybridized to equivalent amounts (1×10^6 cpm/ml) of radiolabeled cDNA derived from the mRNAs of superior cervical ganglia (SCG) from control and anti-NGF-injected rat pups; or, in other experiments, from the mRNAs of control and Aβ 25-35-treated neuronal PC12 cells. cDNA to be used as probe was synthesized from poly(A)+ mRNAs using oligo-dT primers and MMLV reverse transcriptase (Pharmacia) and then radiolabeled to high specific activity as above. Alternatively, radioloabeled cDNA was prepared directly from poly(A)+ mRNA using random hexanucleotide primers, α [^{32}P]-dCTP (NEN Dupont), and AMV reverse transcriptase (Life Technologies) by standard protocols (Sambrook *et al.*, 1989). Amplified products derived from cloned cDNA inserts corresponding to *cyclophillin* were included on blots and were used to standardize signal intensities between duplicate pairs. For each set of experiments (anti-NGF/SCG or Aβ 25-35/PC12), results were confirmed using different sets of blots and different mRNA preparations for cDNA probes.

RT-PCR Analysis

cDNA preparation. Poly(A)+ RNA isolated from SCG of saline-injected (control) or anti-NGF-injected (treated) rat pups was converted to cDNA with Superscript (Life Technologies) using random hexamers (16 μM) as primers. The 35-μl reaction contained 50 m*M* Tris (pH 8.3), 40 m*M* KCl, 6 m*M* MgCl2, 10 m*M* dithiothreitol, 500 μM each dATP, dCTP, dGTP, and dTTP, and 40 U RNasin (Promega Corp., Madison, WI). Samples were incubated at 42°C for 1.5 hr. NaOH was added to 0.3 *M* and reactions were incubated at 60°C for 30 min to hydrolyze RNA templates. After neutralization with HCl, cDNAs were dialyzed against 50 ml of distilled water on floating microfiber filters (Whatman, Hillsboro, OR). Small volume tracer reactions were performed using radiolabeled dCTP to quantify and monitor the quality of the cDNA produced.

PCR reactions. For PCR amplifications of specific cDNAs, 50-μl reactions contained 50 μM dCTP, 100 μM each dATP, dGTP, and dTTP, 10 μCi α-[^{32}P]dCTP (3000 Ci/mmol), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 9), 0.1% Triton X-100, 1 μM each primer, 1 U Taq polymerase, and equal amounts of cDNA templates (typically 0.5–1% of the cDNA synthesized in reverse transcription reactions). Typical reaction conditions were 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C, for 25 cycles. After amplification, cDNAs were separated by electrophoresis on 8% polyacrylamide gels, visualized by autoradiography of dried gels, and quantified with a PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA). Preliminary PCR reactions using primers specific to *GAPDH* sequences served to standardize cDNA template amounts derived from control and treated ganglia, once it was established that *GAPDH* expression did not vary. Reactions using primers specific to cloned genes were repeated three times for each preparation of RNA (from one dissected litter), and at least two different litters were examined for each probe at the 18 and 28 hr time points. Oligonucleotide primers specific for cloned genes were derived from the partial sequences of cloned cDNA inserts as follows:

mud-3: 5′ primer: 5′-GAGCTAGAGCCAGCCAGTCACCCT-TAGTGAG-3′
3′ primer: 5′-GTCTCTTCTGTCGGAATAGCTTAC-AAACTT-3′
mud-5/8: 5′ primer: 5′-AGCAACCACATGGTGGCTCAG-AACCATCTG-3
3′ primer: 5′-CCATGTGTTGCTGGGAATTGACTC-ATGACC-3
mud-7: 5′ primer: 5′-ATGCACAGCACACGTGTTGAGGCT-GACCGC-3′
3′ primer: 5′-GCGCATACAACTCTCCAAGTGTAG-CACTAC-3′

Primer sequences for *mud-5/8* are derived from the cDNA clone for *mud-5* but would detect RNAs corresponding to both *mud-5* and *mud-8*, and probably to other B2-containing sequences. Oligonucleotide primer sequences specific for control genes were as follows:

mouse*cyclin D1* (Matsushime *et al.*, 1991):
5′ primer: 5′-GCGAATTCGATGAAGGAGACCATTCCCT-3′
3′ primer: 5′-GGGGATCCTCTGCTTGTTCTCATCCGC-3′
rat*tyrosine hydroxylase* (*T-OH*) (Grima *et al.*, 1985):
5′ primer: 5′-TTCAGAAGGGCCGTCTCAGA-3′
3′ primer: 5′-CCGCTGCTGCTGCTGCAGCT-3′
rat*S100β* (Kuwano *et al.*, 1986):
5′ primer: 5′-GGGAATTCGGATGTCTGAGCTGGAGAAG-3′
3′ primer: 5′-GCGGATCCACTCCTGGAAGTCACACTCC-3′
rat*GAPDH* (Tso *et al.*, 1985):
5′ primer: 5′-ACCAGGTTGTCTCCTGTGACTTCAACAGC-AAC-3′
3′ primer: 5′-GGGTTTCTTACTCCTTGGAGGCCATGT-AGG-3′

In Situ Hybridization

SCG were dissected from saline-injected or anti-NGF-injected rat pups at designated times, immersed in OCT freezing compound (Miles Inc., Elkhart, IN), quick frozen in methylbutane on dry ice, and stored at −80°C until needed. Cryosections were cut 4-μm thick and thaw-mounted onto gelatin-coated, baked slides. *In situ* hybridization with digoxigenin-RNA probes followed the protocol in Panoskaltsis-Mortari and Bucy (1992), with minor modifications: Slides were fixed in 3% paraformaldehyde in PBS for 1 hr, acetylated with 0.25% acetic anhydride, and hybridized at 50°C overnight with digoxygenin-labeled RNA probes (250 ng/ml) in 50% formamide, 4× SSC, 1× Denhardt's solution (Amersham), 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast RNA, and 10% dextran sulfate. Sense and antisense RNA probes were synthesized using T3 and T7 RNA polymerases (Stratagene) and digoxygenin-UTP as per manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). Sense probes served as specificity controls. *In situ* hybridization results were confirmed on sections from several independent ganglia. Hybridizations with RNA probes corresponding to *neurofilament-M* (*NF-M*) were conducted in parallel as a positive control.

After treatment with ribonuclease and high stringency washes, slides were incubated with alkaline phosphatase-labeled sheep-anti-digoxygenin antibodies (Boehringer Mannheim), and antibodies were detected *in situ* by reaction with substrate solution containing NBT/BCIP (Boehringer Mannheim). After color development, slides were mounted in Gelmount acqueous medium (Biomeda, Foster City, CA). To visualize chromatin in cells, some slides were stained for 10 min in Hoechts 33,258 (1 μg/ml in PBS) before mounting.

RESULTS

Preparing and screening a subtracted cDNA library from differentiated PC12 cells undergoing programmed cell death. To identify genes that may play a role in, or act as markers for, programmed cell death in neurons, we constructed a subtracted cDNA library enriched in sequences expressed at early times after NGF removal from terminally differentiated NGF-dependent PC12 cells (Pittman *et al.*, 1993). These cells become neuronal when cultured in the presence of NGF for 10 days and undergo apoptotic death when NGF is removed. The cell morphology and the timing and mode of NGF-dependent death exhibited by these differentiated PC12 cells closely resembles that of sympathetic neurons in culture; it was our hope, therefore, that this *in vitro* model system would mimic *in vivo* cell death events in these neurons.

The target population for the construction of the subtracted library consisted of neuronal PC12 cells deprived of NGF and cultured in the presence of anti-NGF antibodies and cycloheximide for 6 hr. At this time, cells appear normal morphologically, and DNA laddering is not detected; however, changes in DNA/protein binding patterns are evident, indicating that gene expression patterns already may be changing (Wang and Pittman, 1993). Cells were treated with cycloheximide during NGF withdrawal to allow accumulation of otherwise labile or low-abundance transcripts and to enrich for primary response sequences (expressed without protein synthesis) (Almendral *et al.*, 1988).

The strategy used to construct and screen a ''cell death-enriched'' subtracted library is summarized under Materials and Methods. 2300 recombinants from this enriched library were screened by colony blot hybridization with cDNA probes representing mRNAs from control cells and from cells programmed to die. Before use, the cDNA reverse transcribed from mRNA of programmed cells was subtracted

twice by hybridization with excess sense RNA generated *in vitro* from the control source library. The nonhybridized death-enriched cDNA was radiolabeled to high specific activity by random primer extension and used versus nonsubtracted similarly radiolabeled cDNA derived from control cell mRNA, in a modified version of selective hybridization screening. We considered a recombinant to be positive if it hybridized with the enriched cDNA probe but not at all or very weakly with the cDNA probe derived from control cells. Using this strategy, 200 primary positives were identified. The original duplicate filter sets were then switched and hybridized with the opposite probe for a second screen. Positive clones were picked onto master plates and rescreened by selective colony blot hybridization as before, and by conventional differential hybridization with nonsubtracted cDNA probes derived from control and programmed neuronal PC12 cells. On the basis of these multiple rounds of screening, and cross hybridization studies among positives, 43 unique clones were selected for further study.

Northern blot analysis. To verify that the selected cDNA clones represented induced genes, and to determine the size of their corresponding messages, cDNA inserts were gel purified and used to generate probes for Northern blot analysis. On the basis of this screening step, seven clones designated *message up-regulated during death* (*mud-1* to *mud-7*), exhibiting a moderate to high inducibility in cells programmed to die were chosen for further study (Fig. 1). An eighth cDNA clone, *mud-8*, was found to be >85% identical to *mud-5* and represents another member of the same family of transcribed repetitive elements (see identity of cloned genes below); we therefore grouped these two clones together and refer to sequences representing this family as *mud-5/8*. The mRNA sizes of cloned genes range from 0.35 to 9.5 kilobases (kb), with several of these clones hybridizing to more than one RNA species (see Table 1 for summary). As seen in Fig. 1, two clones, *mud-4* and *mud-6*, each hybridize to two different molecular weight mRNA species; the larger in both cases is the one induced in NGF-deprived cells. *Mud-5* hybridizes to a range of small RNAs centered around 0.35 kb and to a larger 3.2-kb transcript (data not shown); both species are induced in NGF-deprived cells. The remaining cDNAs hybridize to single RNA species.

Expression of cloned sequences during programmed cell death in vivo. Having identified eight cDNAs corresponding to messages induced in neuronal PC12 cells deprived of NGF in the presence of cycloheximide, it was important to determine which of these sequences, if any, had the best chance of playing a role in neuronal cell death. Since a primary goal of this study was to identify genes that functioned in, or, at the very least, could act as markers for, neuronal cell death *in vivo*, we examined expression of the cloned sequences in postnatal rat superior cervical ganglia undergoing programmed cell death (see below). Rat sympathetic ganglia were chosen for the *in vivo* study since neuronal PC12 cells closely resemble primary cultures of sympathetic neurons in both morphology and the mode of death after NGF removal (Martin *et al.*, 1988; Pittman *et al.*, 1993; Deckworth and Johnson, 1993). Detecting induction of any of the sequences in this *in vivo* tissue, therefore, would not only identify clones of top priority, but also help validate neuronal PC12 cells as a model for *in vivo* cell death events in sympathetic neurons and possibly other types of neurons as well.

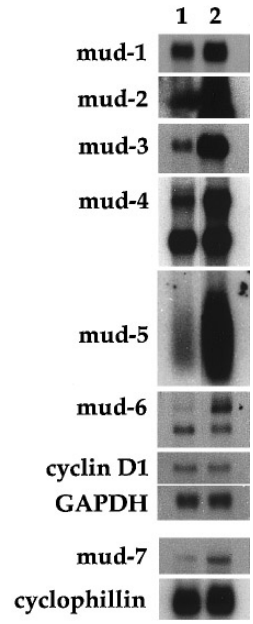

FIG. 1. Cloned sequences are induced in neuronal PC12 cells programmed to die by NGF deprivation. Northern blot analysis of polyadenylated mRNA (5 μg/lane) from control neuronal PC12 cells (lane 1) and "programmed" cells deprived of NGF for 6 hr (lane 2). Blots were hybridized with radiolabeled probes corresponding to *muds 1–7*, *cyclin D1*, and control sequences *GAPDH* and *cyclophillin*. The "programmed" RNA for blots probed with *mud-7* and *cyclophillin* sequences was derived from cells deprived of NGF in the absence of cycloheximide. In all other blots, "programmed" RNA was derived from cells deprived of NGF in the presence of 10 μg/ml cycloheximide.

Naturally occuring cell death in rat sympathetic ganglia results in the loss of nearly 35% of the neuronal population, but since it proceeds over 3 days from Postnatal Days 3–7, only a small number of cells are dying at any one time (Wright *et al.*, 1983). To increase the number of dying sympathetic neurons in the population, and to improve the constrast between control (living) and experimental (dying) ganglia, naturally occurring cell death was enhanced by injecting 1-day-old rat pups with antibodies to NGF, as previously described (Levi-Montalcini and Angeletti, 1966). Dissections at 72 hr posttreatment revealed degenerated sympathetic ganglia containing few neurons. Sympathetic ganglia dissected 48 hr after anti-NGF injection were visibly deteriorated compared to control ganglia and were judged to be too advanced for testing gene expression. At 12–28 hr postinjection, ganglia were intact, and neuronal morphology was very good. Therefore, sympathetic ganglia dissected

TABLE 1
Summary of Potential Cell Death-Induced cDNAs

Sequence	cDNA (kb)	mRNA (kb)	Induction SCG (anti-NGF) Sblot	Induction SCG (anti-NGF) RT-PCR	Induction PC12 (Aβ 25-35) Sblot
mud-1	0.4	2.6	+		=
mud-2	0.6	1.1	=		=
mud-3	0.5	2.8	nd	++	=
mud-4	0.55	4.5	=		=
		2.6			
mud-5/8	0.35	0.35	=	++++	++++
		3.2			
mud-6	0.85	5.4	++		++
		4.4			
mud-7	0.5	9.5	nd	++	=

Note. Expression summary of potential cell death-induced cDNAs. mRNA sizes of cloned cDNAs were estimated by comparison to RNA size standards on Northern blots. Sequences testing positive for induction by reverse Southern blot (Sblot) or RT-PCR *in vivo* in sympathetic ganglia deprived of NGF (anti-NGF), or by reverse Southern blot *in vitro* in neuronal PC12 cells exposed to neurotoxic amyloid peptide (Aβ 25-35) are indicated in bold-face type. +, relative induction in dying cells. =, equivalent expression in dying and control cells. nd, not detected in either dying or control cells. Results are the same for both *mud-5* and *mud-8* cDNAs, which are grouped together as two nearly identical B2-transcribed repeats.

between 12 and 28 hr postinjection were chosen for testing expression of cloned sequences. Since the amount of tissue available from sympathetic ganglia is limiting, this expression survey was conducted using reverse Southern blot analysis (see Materials and Methods). Figure 2 shows duplicate Southern blots of the cloned cDNA inserts hybridized to radiolabeled cDNA derived from the mRNA of control ganglia injected with saline (Fig. 2A), and of treated ganglia injected with antibodies to NGF (Fig. 2B). In both cases, ganglia were dissected 28 hr postinjection. Most *mud* sequences show their corresponding mRNAs to be expressed nearly equally in both control and treated ganglia. In contrast, *mud-1* and *mud-6* correspond to moderately induced mRNAs, indicating that some of the sequences may be upregulated in trophic factor-dependent cell death *in vivo*. It should be noted that because *mud-4* hybridizes to two different RNAs (see Fig. 1), only one of which is induced in NGF-deprived neuronal PC12 cells, it is possible that a similar induction occurs in sympathetic ganglia but is obscured in these experiments.

Since we failed to detect in either set of ganglia mRNAs corresponding to *mud-3* and *mud-7*, it seemed possible that these messages were present, but too low in abundance for analysis by reverse Southern blot. Therefore, expression of selected cDNA sequences was examined in control and treated ganglia using the reverse transcription polymerase chain reaction (RT-PCR). Although the amplification protocol used was not aimed at obtaining quantitative data, it permitted a relative comparison of particular mRNA levels among different tissue samples (see Materials and Methods). mRNA was prepared from superior cervical ganglia dissected 28 hr postinjection from control (saline-injected) and 12, 18, or 28 hr postinjection from treated (anti-NGF-injected) rat pups and used as the template for RT-PCR amplification of specific sequences (Fig. 3). Oligonucleotide primers specific for cloned genes were derived from partial sequences of cloned cDNA inserts (see Materials and Methods). mRNAs corresponding to *mud-3* and *mud-7* were detectable by this method, supporting the idea that these were low abundance messages. Most importantly, both *mud-3* and *mud-7* are up-regulated in ganglia undergoing cell death. Expression of *cyclin D1*, previously shown to be induced in primary cultures of sympathetic neurons undergoing PCD (Freeman *et al.*, 1994) and therefore chosen as a potential positive control, is induced in these experiments

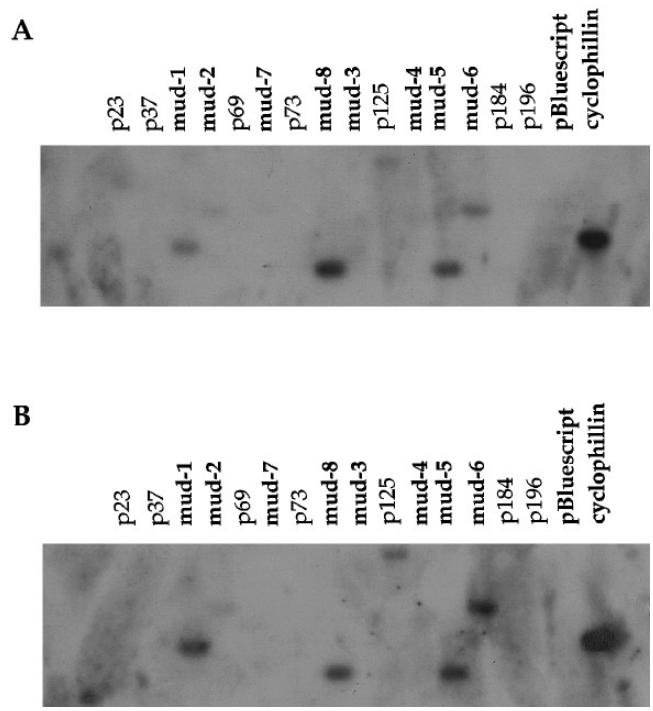

FIG. 2. Induction of cloned sequences in superior cervical ganglia (SCG) undergoing programmed cell death: reverse Southern analysis. Duplicate Southern blots of cloned cDNA inserts were hybridized to radiolabeled cDNA probe derived from RNA of SCG dissected 28 hr posttreatment from saline-injected rat pups (A) or from anti-NGF antibody-injected rat pups (B). Inserts corresponding to *muds 1–8*, and to control sequence *cyclophillin*, are indicated. Other cloned cDNAs, isolated in this screen but not pursued in this study, are also present on blots (indicated by clone number). Signal densities for cloned cDNAs were compared between duplicate blots, after standardization to signal densities obtained for control *cyclophillin*. Results were confirmed on a second set of blots using mRNA from an independent set of dissections as the source of cDNA probe.

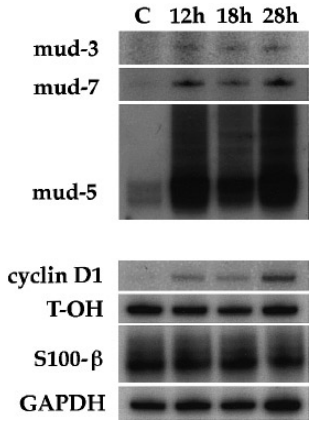

FIG. 3. Induction of cloned sequences in superior cervical ganglia (SCG) undergoing programmed cell death: RT-PCR analysis. Polyadenylated mRNA from SCG dissected 28 hr posttreatment from saline-injected (Control) and 12, 18, and 28 hr posttreatment from anti-NGF antibody-injected (12h, 18h, 28h) rat pups was converted to cDNA, and equal amounts were analyzed by PCR amplification using primers specific to the sequences indicated. Primers derived from *mud-5* cDNA will also detect *mud-8* B2 transcripts. *cyclin D1* sequences were tested as a potential positive control; *GAPDH, T-OH,* and *S100β* sequences served as uninduced controls and cellular marker genes. Data presented here are from a single preparation of ganglia. Each gene induction was confirmed in at least two independent ganglia preparations.

as well, demonstrating for the first time the induction of this gene in PCD *in vivo*. The expression of three different control sequences was also examined (bottom panels). Expression of neuron-specific marker *tyrosine hydroxylase* (*T-OH*), Schwann cell marker *S100β,* and ubiquitous gene *GAPDH,* respectively, remains relatively constant in control and treated ganglia, and is directly proportional to input template amounts. Thus, no major loss of neurons or decline in neuronal RNA synthesis occurs up to 28 hr after injection of anti-NGF antibodies.

The only discrepancy between the two methods of assaying RNA expression in the ganglia (reverse Southern blot and RT-PCR), was seen with *mud-5/8* sequences. Whereas by reverse Southern (Fig. 2) *mud-5/8* sequences do not appear up-regulated in treated versus control ganglia, by RT-PCR (Fig. 3), multiple bands containing *mud-5/8*-hybridizing sequences reflect robust increases in corresponding cDNA templates in treated versus control ganglia. A possible explanation for this became apparent when *mud-5/8* sequences were determined to correspond to rodent B2 repetitive element-containing transcripts, of which there are two populations in the cell: nuclear B2-containing hnRNA or snRNAs and small cytoplasmic poly(A)+ B2 RNAs (see identity of cloned genes below, and Discussion). Whereas both nuclear and cytoplasmic B2-homologous RNAs would easily be detected in the random oligomer-primed cDNA template used for RT-PCR by extension with B2-specific primers, only the cytoplasmic poly(A)+ species would be easily detected by reverse Southern using a complex, oligo dT-primed cDNA probe. Thus, B2-containing nuclear or nonpolyadenylated RNAs but not small B2 poly (A)+ RNAs, may be induced in NGF-deprived ganglia. Preliminary *in situ* hybridizations using *mud-8* as a probe are consistent with the induction of nuclear B2-containing species in the neurons of NGF-deprived SCG (data not shown).

The results shown in Figs. 2 and 3 identify five sequences, represented by *mud-1, mud-3, mud-5/8, mud-6,* and *mud-7,* as genes up-regulated in sympathetic ganglia programmed to die, and therefore, as potential candidates for a role in neuronal cell death *in vivo*. To help exclude the possibility that the induction of these cDNAs is due to some effect of NGF deprivation other than cell death, sequences were screened for expression in neuronal PC12 cells triggered to

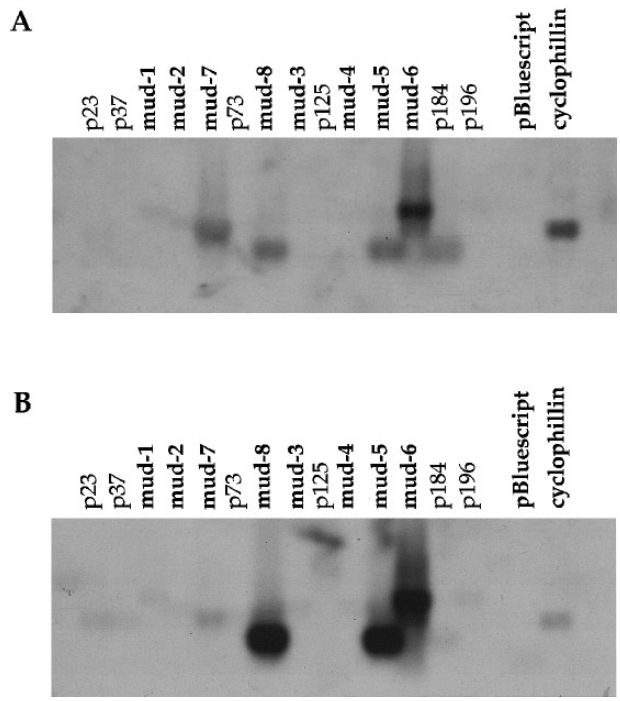

FIG. 4. Several cloned sequences are induced in neuronal PC12 cells programmed to die by exposure to neurotoxic b amyloid peptide Aβ 25–35. Duplicate Southern blots of cloned cDNA inserts were hybridized to radiolabeled cDNA probe derived from RNA of control neuronal PC12 cells (A) or neuronal PC12 cells exposed to 25 μM Aβ 25–35 for 24 hr (B). Inserts corresponding to *muds 1–8,* and to control sequence *cyclophillin,* are indicated. Other cloned cDNAs, isolated in this screen but not pursued in this study, are also present on blots and indicated by clone number. Signal densities for cloned cDNAs were compared between duplicate blots, after standardization to signal densities obtained for *cyclophillin.* Note that autoradiogram in B is purposely underexposed (compare cyclophillin signals) so that large signals for *mud 5* and *mud 8* do not obscure other data. Results were confirmed on a second set of blots using independent mRNA preparations for cDNA probes.

die by exposure to the neurotoxin Aβ 25-35 (Pike *et al.*, 1993), in the presence of NGF. Aβ 25-35 triggers transcription-dependent apoptotic death in neuronal PC12 cells in the presence of NGF over the course of 24–48 hr (S. Wang, unpublished results). Figure 4 shows duplicate Southern blots of the cloned cDNA inserts hybridized to radiolabeled cDNA derived from the mRNA of control neuronal PC12 cells (Fig. 4A), and of neuronal PC12 cells treated with Aβ 25-35 for 24 hr (Fig. 4B). *mud-1*, *mud-2*, *mud-3*, and *mud-4* show little or no induction in this paradigm, and *mud-7* shows a slight decrease. However, *mud-5*, *mud-6*, and *mud-8* show a robust up-regulation of corresponding mRNAs in treated cells, indicating that accumulation of these transcripts is responsive to a death trigger other than removal of NGF, and in fact occurs in the presence of NGF. Several sequences (*mud-3*, *mud-5*, and *mud-7*) were also examined for induction in PC12 cells following a necrotic insult (2.5 hr in 20 m*M* 2-deoxyglucose and 20 μM rotenone) and showed little to no change in expression, indicating the genes were not responding in a nonspecific manner to cell stress (data not shown). These results support the notion that the induction of at least some of the cloned sequences, including two up-regulated *in vivo* in sympathetic ganglia (*mud-6* and *mud-5/8*), is specific to the cell death process. In addition, induction of cloned sequences in either NGF-deprived SCG or Aβ 25-35-treated neuronal PC12 cells demonstrates that these genes (*mud-1*, *mud-3*, *mud-5/8*, *mud-6*, *mud-7*), although originally cloned in the presence of cycloheximide to allow accumulation of low abundance transcripts and to enrich for primary response genes, do not require cycloheximide for their induction.

Expression of induced sequences in situ during cell death. Since the amount of mRNA available for biochemical studies from rat superior cervical ganglia is limiting, reverse Southern blot and RT-PCR techniques were used until this point to analyze the expression of cloned cDNAs *in vivo*. In order to confirm the results obtained with these methods, and to visualize the pattern of expression *in vivo*, we examined by *in situ* hybridization the expression of three cDNA clones identified as sequences induced in sympathetic ganglia undergoing cell death. We chose to test *mud-3*, *mud-6*, and *mud-7* sequences because they were most strongly induced from relatively low control levels, and because they represented unique (not repetitive) genes (see next section).

Frozen sections of sympathetic ganglia dissected 24–28 hr postinjection from control (saline-injected) and treated (anti-NGF injected) rat pups were hybridized *in situ* to dioxygenin-labeled RNA probes derived from cloned cDNA inserts (Fig. 5). *Neurofilament* (*NF-M*) mRNA is found in equivalent amounts in both control and treated ganglia (Figs. 5A and 5B) and is localized in the cytoplasm of sympathetic neurons in the ganglia (larger cells). *mud-3*, *mud-6*, and *mud-7* sequences (Figs. 5C and 5D; 5E and 5F; and 5G and 5H; respectively) are also localized in the cytoplasm of sympathetic neurons, but in contrast to *NF-M* RNA, all three transcripts are up-regulated in ganglia undergoing cell death compared to control ganglia. It should be noted that the *mud-6* probe used here hybridizes to both mRNAs associated with this clone (see Table 1), so it is not clear which message is up-regulated *in situ*. However, since this sequence was cloned on the basis of the induction of the larger 5.4-kb RNA in neuronal PC12 cells, it is likely that the same transcript is responsible for the increase in steady state levels of *mud-6* sequences seen here. Induction of these mRNAs occurs in nearly all neurons of NGF-deprived sympathetic ganglia, the overwhelming majority of which at this time contain nuclei with normal, uncondensed chromatin, as visualized by Hoescht's staining (Fig. 6). Thus, genes are induced early, before the onset of major morphological changes characteristic of apoptosis, a result consistent with our cloning strategy to target early response genes.

These *in situ* hybridization studies confirm results obtained by reverse Southern (*mud-6*) and by RT-PCR (*mud-3*, *mud-7*) with respect to up-regulation of cloned sequences in dying ganglia. In addition, they show that expression is localized in neurons, consistent with the notion that the cloned genes are involved in, or can act as markers for, neuronal cell death *in vivo*.

Preliminary sequence data and identification of cDNA clones. To determine whether the cloned cDNA sequences represent novel, or previously described genes, partial sequences were obtained from both ends of each cDNA insert and compared with known DNA sequences in GenBank. Although only *mud-1*, *mud-3*, *mud-5/8*, *mud-6*, and *mud-7* have been shown to be up-regulated *in vivo* in sympathetic neurons, we include sequence data on *mud-2* and *mud-4*, since they have not been ruled out as cell death-related genes. Six of eight clones (*mud-1*, *mud-3*, *mud-4*, *mud-5*, *mud-6*, *mud-8*) were found to be either identical or similar to previously identified genes (Table 2). A comparison of approximately 300 nucleotides near the 3′ end of *mud-1* with sequences in GenBank detected 100% identity with the gene for rat annexin VI, a calcium-dependent membrane-associated protein (Fan *et al.*, 1995). Similarly, more than 300 nucleotides from *mud-3* reveal identity with rat NGF early response gene *PC3* (Bradbury *et al.*, 1991), the homolog of mouse gene *TIS21* (Fletcher *et al.*, 1991). *TIS21* has recently been identified among a panel of preselected early response genes as one that is induced in cell death of neuronal PC12 cells and fibroblasts (Mesner *et al.*, 1995). Database searches with over 300 nucleotides (nearly the size of the detected mRNA) from *mud-5* revealed 85–90% identity with mouse B2 repetitive sequence mRNA, suggesting that this clone corresponds to a rat homolog of these sequences (Krayev *et al.*, 1982). The B2 family of repetitive elements is widely dispersed throughout the mouse genome, and sequences from this family have been found both in coding and noncoding regions of larger genes, as well as by themselves in a class of small heterogenous poly(A)+ mRNAs transcribed by RNA polymerase III. *mud-8*, which shares >85% identity with *mud-5*, is another small poly(A)+ RNA member of the B2 family in rat. Sequence analysis of *mud-6* using over 200 bases from mainly the 3′UTR of the cDNA insert reveals 64–91%

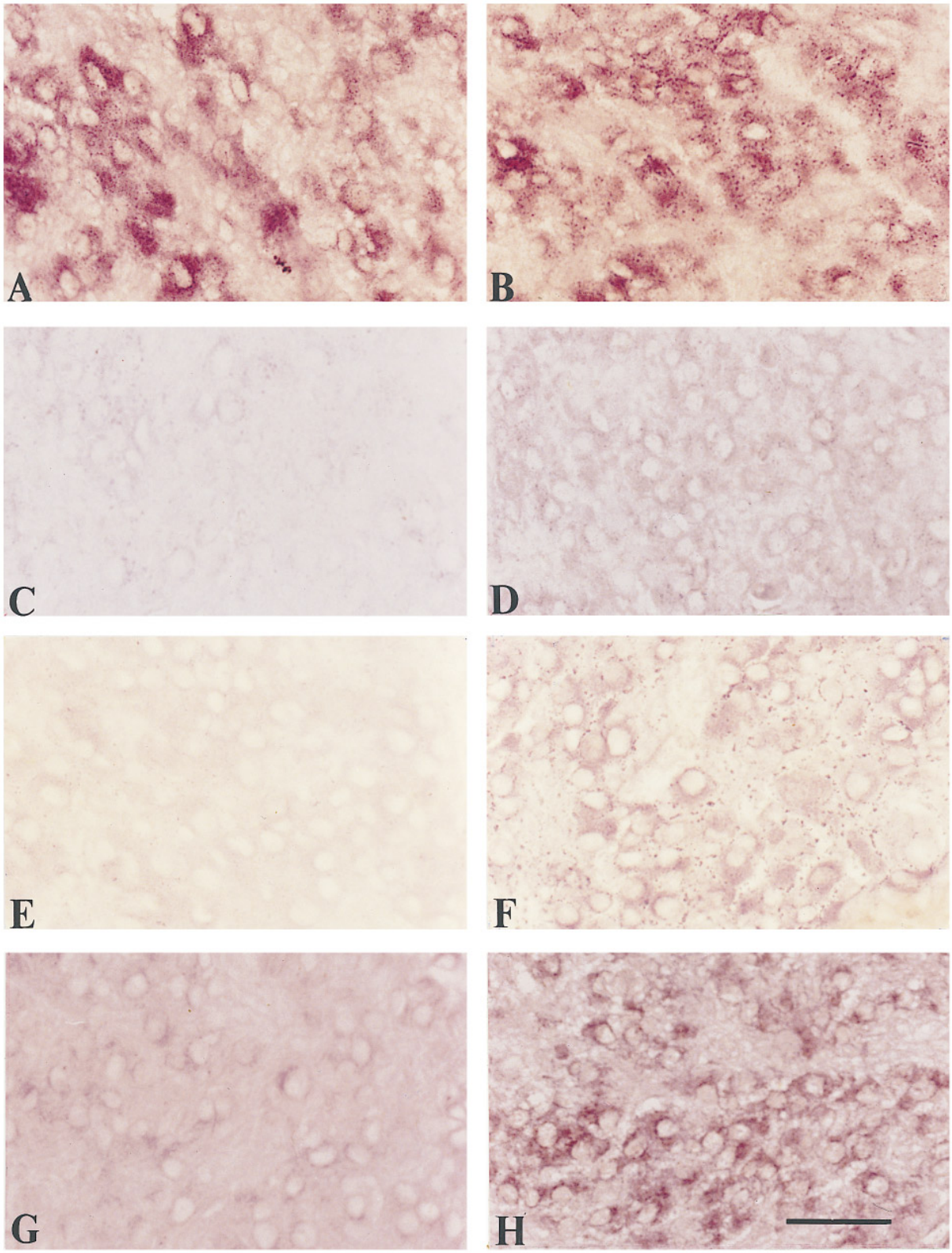

FIG. 5. *Mud* sequences are induced *in situ* in neurons of sympathetic ganglia undergoing PCD. Bright field photomicrographs showing hybridization *in situ* to frozen sections of sympathetic ganglia dissected 24 hr after treatment from control rat pups (A, C, E, G), or pups injected with antibodies against NGF (B, D, F, H). Digoxygenin-labeled RNA probes corresponding to neurofilament-M (A and B), *mud-3* (C and D), *mud-6* (E and F), and *mud-7* (G and H) are shown. Results were confirmed in sections from several independent ganglia. Bar, 50 μm.

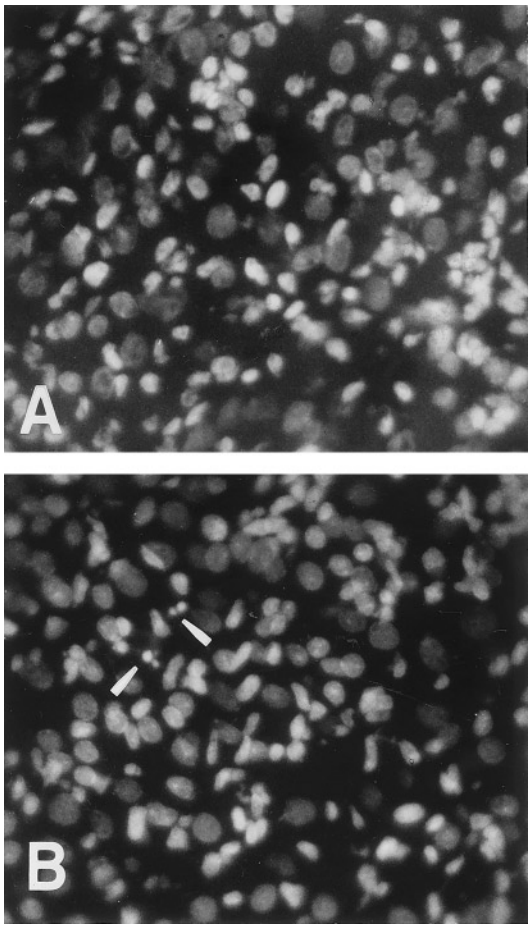

FIG. 6. Chromatin integrity of the overwhelming majority of sympathetic neurons from both control and NGF-deprived ganglia remains intact. Frozen sections of sympathetic ganglia dissected 24 hr after treatment from control rat pups (A) or pups injected with anti-NGF (B) were fixed and stained with Hoechst 33,258, and viewed by fluorescence microscopy. Apoptotic nuclei are indicated by the white arrowheads.

sequence identity to *Ring3* (Beck *et al.*, 1992), the human homolog of the Drosophila *female sterile homeotic* (*fsh*), a positive regulator of the *bithorax* complex (Haynes *et al.*, 1989). This suggests that *mud-6* corresponds to the rat homolog of these genes. Finally, comparison of over 200 nucleotides from *mud-4* with GenBank sequences detected 60–80% identity with human TAFII70 mRNA, encoding one of the TATA box-binding protein-associated factors (Weinzieri *et al.*, 1993), suggesting *mud-4* corresponds to the rat homolog of this gene. Searches of GenBank with sequences from *mud-2* (350 nucleotides) detected no significant similarities to known genes. Partial sequence data for *mud-7* from overlapping lambda ZAP clones of the programmed library totaled over 2000 bases, with no significant similarities detected.

DISCUSSION

Strategy for Cloning Cell Death Induced Sequences

A primary goal of this study was to use an *in vitro* model system to identify genes potentially involved in programmed cell death of neurons *in vivo*. In particular, we wanted to target early, possibly regulatory, sequences that mediate entry into the death program, rather than effectors that execute final cell suicide. By constructing a subtracted cDNA library using neuronal PC12 cells deprived of NGF for only 6 hr, and cultured in the presence of cycloheximide, our cloning strategy enriched for rare or labile mRNAs expressed early in the cell death process without prior protein synthesis (primary response genes). At 6 hr, nearly all cells can be rescued by readdition of NGF; thus, any ''death'' genes induced during this time represent precommittment steps in the cell death process—that is, reversible events between the initial trigger (removal of NGF) and the synthesis of effector molecules that irreversibly commits the cell to die. Still, it is not certain that genes cloned as primary response genes in neuronal PC12 cells, and induced in NGF-deprived sympathetic neurons *in vivo*, act as primary response genes in the latter case. As in neuronal PC12 cells, however, genes induced in sympathetic ganglia are ''early'' in that induction occurs in neurons prior to chromatin condensation and prior to any major loss of neurons or decline in neuronal RNA synthesis in the ganglia, as assessed by the expression of various marker genes.

Screening selected cDNAs from the subtracted library for expression in rat SCG undergoing programmed cell death allowed us to identify the best candidates for an *in vivo* role, which were then tested for neuronal expression by *in situ* hybridization. Taken together, the reverse Southern and RT-PCR experiments identify *mud-1*, *mud-3*, *mud-5/8*, *mud-6*, and *mud-7* as the first genes shown to be induced during neuronal PCD of sympathetic ganglia *in vivo*. That five of seven unrelated sequences cloned in dying neuronal PC12 cells are induced in dying sympathetic ganglia *in vivo* is remarkable in that it implies very similar molecular pathways in the two systems. Gene inductions common to the two systems may identify an expression pattern diagnostic of neuronal PCD. It would be interesting to examine the expression *in vivo* in sympathetic ganglia of other death-associated sequences identified in cultures of neuronal PC12 cells (Mesner *et al.*, 1995).

In situ analysis shows that *mud-3*, *mud-6*, and *mud-7* sequences are induced in neurons of sympathetic ganglia at 24 hr post-anti-NGF treatment, supporting the idea that the genes may be involved in the cell death process *in vivo*. Gene induction occurs in nearly all neurons of the ganglia, the majority of which have normal nuclei with uncondensed chromatin and so have not undergone major morphological changes characteristic of apoptosis. Nevertheless, nearly all neurons in treated ganglia are destined to die. Thus, induction occurs at an early stage of the death pathway, and perhaps reflects an initial, simultaneous response of all neurons to the cessation of NGF signal trans-

TABLE 2
Identity of cDNA Clones

cDNA	Sequence identity	Sequence similarity	Accession No.
mud-1	rat *annexin VI*		
mud-2	novel		U70266
mud-3	rat *PC3*	mouse *T1S21*	
mud-4		human *TAFII70*	U70269
mud-5/8		mouse B2 element	U70265/U7030
mud-6		human *Ring 3;* Drosophila *fsh*	U70306
mud-7	novel		U70267

Note. Identity of cDNA clones. Partial nucleotide sequence data from each cDNA clone was compared to sequences in GenBank, and identities or high-scoring similarities with known genes are indicated (see Results). Accession numbers are given for newly cloned rat sequences. *Mud* sequences induced *in vivo* in sympathetic ganglia undergoing PCD are in boldface type.

duction. An early, pan-neuronal induction similar to what is seen here has been observed for *c-jun,* which is necessary for PCD of sympathetic neurons in culture (Estus *et al.,* 1994; Ham *et al.,* 1995).

Death-Responsive Genes in Neuronal PCD

Recent studies examining expression of preselected genes in neuronal PCD describe a cascade of gene induction during apoptosis triggered by the withdrawal of NGF (Freeman *et al.,* 1994; Estus *et al.,* 1994; Ham *et al.,* 1995; Mesner *et al.,* 1995). The present study confirms some of these results and expands the cadre of genes associated with neuronal cell death. First, the reisolation of *PC3/TIS 21* in this screen, and the expression of *mud-3* (*PC3/TIS21*) and *cyclin D1* in the RT-PCR experiments, confirm the induction of these genes during neuronal PCD (Mesner *et al.,* 1995; Freeman *et al.,* 1994) and demonstrate for the first time their induction in sympathetic ganglia undergoing cell death *in vivo.* That other death-induced genes identified in previous studies (Mesner *et al.,* 1995; Estus *et al.,* 1994) were not reisolated in this screen is not entirely surprising since they may be induced too late in the death cascade for detection by our cloning strategy (e.g., *c-fos, fos B, rhl, jun B, NGF1-A, TIS 11*), or they may not be induced in common between PC12 cells and sympathetic neurons (e.g., *mkp-1, c-myb, fos B, rhl, jun B*). Clearly, however, this subtractive hybridization screen was not an exhaustive one since *c-jun* might have been, but was not, reisolated by us.

Second, a new set of induced genes associated with neuronal cell death has been identified and includes: the rat homologs of human *Ring3* and Drosophila homeotic regulator *fsh* (Haynes *et al.,* 1989; Beck *et al.,* 1992), human transcription factor *TAFII70* (Weinzieri *et al.,* 1993), and mouse B2 repetitive element-containing transcripts (Krayev *et al.,* 1982), rat Ca^{2+}-dependent phospholipid binding protein *annexin VI* (Fan *et al.,* 1995), and two novel sequences. Of these, *mud-1* (*annexin VI*), *mud-5/8* (B2 repeats), *mud-6* (*Ring3/fsh*), and *mud-7* (novel), along with *mud-3* (*PC3/TIS21*), are the strongest candidates for a role in neuronal PCD since their induction was detected *in vivo* in NGF-deprived sympathetic ganglia. Interestingly, the cellular distribution of annexin VI is altered in neurons under pathological conditions and is associated with granulovacuolar bodies in Alzheimer's disease (Eberhard *et al.,* 1994).

In considering the identity of the cloned sequences apropó of cell death, two themes present themselves. The first involves the control of global transcription and is given by considering *mud-6* (*Ring3/fsh*). Proteins encoded by *Ring3* and *fsh* (and by extension *mud-6*) feature a conserved motif, the bromodomain, implicated in protein–protein interactions (Haynes *et al.,* 1989, 1992). All known bromodomain-encoding proteins are involved in some aspect of transcriptional control (e.g., Gansheroff *et al.,* 1995; Marcus *et al.,* 1994; Peterson and Herskowitz, 1992; Lygerou *et al.,* 1994; Tamkun *et al.,* 1992; Ruppert *et al.,* 1993; Randazzo *et al.,* 1994; Eckner *et al.,* 1994). Bromodomain proteins may be needed for the expression of broad classes of genes (Chiba *et al.,* 1994; Lygerou *et al.,* 1994) and appear to influence the assembly of multicomponent complexes involved in transcriptional activation, either by bridging classic sequence-specific upstream activators and the TFIID complex or by derepressing chromatin (Hirschhorn *et al.,* 1992; Lygerou *et al.,* 1994; Randazzo *et al.,* 1994). In Drosophila, *fsh* functions as a positive regulator of the homeotic *bithorax* complex, via its interaction with genes of the *trithorax* group (*trx-G*) (Digan *et al.,* 1986; Shearn, 1989). One member of *trx-G, brahma* (a bromodomain protein), is a known antagonizer of chromatin repression (Tamkun *et al.,* 1992); another member, *ash-1,* is thought to encode the Drosophila homolog of h*TAFII70,* d*TAFII60* (Weinzieri *et al.,* 1993). Since different combinations of cofactors (like TAFs or bromodomain-containing proteins) may form subcomplexes of the general transcription machinary that mediate gene activation by different classes of sequence-specific DNA-binding proteins (Goodrich and Tijan, 1994; Dikstein *et al.,* 1996; Hayashida *et al.,* 1994), then perhaps the modulation of one or more cofactors could change the specificity of a transcription complex so that it mediates, for instance, the activation of a set of genes that initiates cell death. Splice variants of cofactors might also change the specificity of a multicomponent transcription complex. In fact, *mud-6*

sequences hybridize to two distinct RNA species, only the larger of which is death-induced. Recent reports of protein–protein interactions involving bromodomains such as TAFII250 (bromodomain)-E1A or -pRb (Geisburg *et al.*, 1995; Shao *et al.*, 1995), and BRG-1(bromodomain)-pRb (Dunaief *et al.*, 1994), suggest that such cofactors are important targets for growth regulators, tumor suppressors, and oncogenes—providing a mechanism for transcriptional control of a set of genes with related functions, such as those involved in PCD.

A second theme, suggested by the identity of *mud-5/8* (B2 elements), involves polymerase III transcription and the role of short interpersed repetitive sequences (SINES) in the control of gene expression. Small B2 RNAs are known polymerase III transcripts; polymerase III-competence might also be conferred on larger hnRNAs and mRNAs that contain copies of B2 repeats (Kramerov *et al.*, 1985; Rigby *et al.*, 1985). Thus the fact that neuronal PC12 cells triggered to die by either NGF withdrawal or exposure to neurotoxic peptide Aβ 25-35, and sympathetic neurons undergoing PCD, exhibit a substantial increase in the abundance of B2-containing RNAs, may reflect a specific induction of polymerase III-dependent B2 transcripts, or an increase in a more general activity of RNA polymerase III. Interestingly, specific induction of pol III-transcribed Alu elements has been shown to occur via the unmasking of certain repressed chromosomal sites (Russanova *et al.*, 1995), providing a scenario whereby the actions of *mud-6* and *mud-5/8* might converge. Induction of B2 RNAs has been associated with heat shock (Fornace and Mitchell, 1986), serum stimulation (Edwards *et al.*, 1985), viral transformation (Rigby *et al.*, 1985), and transition from a quiescent to proliferative state (Lania *et al.*, 1987), while significant decreases of B2 RNAs are seen with differentiation in embryonal carcinoma or implantation development of mouse embryos (White *et al.*, 1989; Bachvarova, 1988). A role for small B2 RNAs in splicing is suggested by their association with hnRNAs and snRNPs in the nucleus (Krayev, 1982), while a role in control of mRNA translation or half-life is suggested by association with mRNA and RNPs in the cytoplasm (Kramerov *et al.*, 1985; Konstantinova *et al.*, 1995). B2 sequences forming part of particular hnRNAs also may act as alternative splicing subtrates (Pattanakitsakul *et al.*, 1992). Other SINE-homologous transcribed sequences have been linked to tissue-specific or developmentally regulated gene expression, including the ID repeat in neural tissue (McKinnon *et al.*, 1987), the opa repeat originally found in the neurogenic locus Notch (Wharton *et al.*, 1985), and Alu repetitive sequences (Watson and Sutcliffe, 1987). Taken together, the identities of *mud-6* and *mud-5/8* suggest that entry into the death pathway may entail changes in general transcription and/or splicing machinary and genomic structure, as well as expected changes in the complement of sequence-specific transcription factors.

Finally, as is true of death-associated genes previously identified, several of the genes isolated in this study suggest a connection with some aspect of proliferation and/or differentiation control. The homolog of *mud-6, fsh,* controls segment formation and identity in Drosophila (Digan *et al.*, 1986); mouse homologs of *mud-5/8* (B2 repeat) are expressed in a growth and differentiation-dependent manner; *mud-3* (PC3/TIS21) is a member of a gene family of cell cycle regulated, anti-proliferative molecules (Rouault *et al.*, 1992); and *mud-1* (*annexin VI*) undergoes growth-dependent posttranslational modification, partially reverses the transformed phenotype of carcinoma cells (Edwards and Moss, 1995), and may be the gene responsible for Treacher Collins syndrome, a craniofacial developmental disorder (Dixon *et al.*, 1994). One hypothesis concerning this apparent death/proliferation/differentiation connection is that cell death is the outcome of conflicting growth-regulatory signals, i.e., the death pathway is enacted when the combination of genes expressed in a particular cell context fails unambiguously to signal cell cycle entry, or arrest, or exit to terminal differentiation (for reviews: Ucker, 1991; Rubin *et al.*, 1993; DiBenedetto and Pittman, 1995).

In summary, the results presented here demonstrate the use of an *in vitro* PC12 cell system to clone and identify a new set of candidate genes for involvement in neuronal PCD *in vivo*. It remains to be seen whether any of these gene products are necessary or sufficient for neuronal cell death in development. The gene inductions identified here, however, are likely to reflect, directly or indirectly, intracellular mechanisms and regulatory events that connect the initial death trigger with the actual death machinary that effects neuronal cell death. As such, their functions will be informative about the suicide process and may serve to expand our concept of apoptosis control.

ACKNOWLEDGMENTS

Research supported in part by NIH Grant NS32465 to Randall N. Pittman and NIH Postdoctoral Fellowship NS09432 to Angela J. DiBenedetto.

REFERENCES

Abbadie, C., Kabrun, N., Bouali, F., Smardova, J., Stehelin, D., Vandenbunder, B., and Enrietto, P. J. (1993). High levels of c-*rel* expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells *in vitro*. *Cell* **75,** 899–912.

Almendral, J. M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol. Cell. Biol.* **8,** 2140–2148.

Bachvarova, R. (1988). Small B2 RNAs in mouse oocytes, embryos and somatic tissues. *Dev. Biol.* **130,** 513–523.

Beck, S., Hanson, I., Kelly, A., Pappin, D. J. C., and Trowsdale, J. (1992). A homologue of the *Drosophila female sterile homeotic* (*fsh*) gene in the class II region of the human MHC. *J. DNA Seq. Map* **2,** 203–210.

Bradbury, A., Possenti, R., Shooter, E. M., and Tirone, F. (1991). Molecular cloning of PC3, a putatively secreted protein whose

mRNA is induced by nerve growth factor and depolarization. *Proc. Natl. Acad. Sci. USA* **88,** 3353–3357.

Chiba, H/, Muramatsu, M/, Nomoto, A. and Kato, H. (1994). Two human homologues of *Saccharomyces cerevisiae SWI2/SNF2* and *Drosophila brahma* are transcriptional activators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucleic Acids Res.* **22,** 1815–1820.

Chomoczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Deckwerth, T. L., and Johnson, E. M. (1993). Temporal analysis of events associated with programmed cell death (Apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* **123,** 1207–1222.

DiBenedetto, A. J., and Pittman, R. N. (1995). Death in the Balance. *Perspec. Dev. Neurobiol.* **3,** 109–117.

Digan, M. E., Haynes, S. R., Mozer, B. A., Dawid, I. B., Forquignon, F., and Gans, M. (1986). Genetic and molecular analysis of *fs(1)h,* a maternal effect homeotic gene in*Drosophila. Dev. Biol.* **114,** 161–169.

Dikstein, R., Zhou, S., and Tijan, R. (1996). Human TAFII105 is a cell type-specific TFIID subunit related to hTAFII130. *Cell* **87,** 137–146.

Dixon, J., Gladwin, A. J., Loftus, S. K., Riley, J. H., Perveen, R., Wasmuth, J. J., Anand, R., and Dixon, M. J. (1994). A YAC contig encompassing the Treacher Collins syndrome critical region at 5q31.3–32. *Ann. J. Hum. Genet.* **55,** 372–378.

Dragunow, M., Young, D., Hughes, P., MacGibbon, G., Lawlor, P., Singleton, K., Sirimanne, E., Beilharz, E., and Gluckman, P. (1993). Is c-*jun* involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? *Mol. Brain Res.* **18,** 347–352.

Dunaief, J. L., Strober, B. E., Guha, S., Khavari, P. A., Alin, K., Luban, J., Begemann, M., Crabtree, G. R., and Goff, S. P. (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell* **79,** 119–130.

Eberhard, D. A., Brown, M. D., and VandenBerg, S. R. (1994). Alterations of annexin expression in pathological neuronal and glial reactions. Immunohistochemical localization of annexins I, II (p36 and p11 subunits), IV and VI in the human hippocampus. *Am. J. Pathol.* **145,** 640–649.

Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994). Molecular cloning and functional analysis of the adenovirus E1A-associated 300kD protein (p300) reveals a protein with properties of a transcriptional adaptor *Genes Dev.* **8,** 869–884.

Edwards, D. R., Parfett, C. L. J., and Denhardt, D. T. (1985). Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: equivalence of one species to B2 repetitive elements. *Mol. Cell. Biol.* **5,** 3280–3288.

Edwards, H. C., and Moss, S. E. (1995). Functional and genetic analysis of annexin VI. *Mol. Cell. Biochem.* **149–150,** 293–299.

Estus, S., Zaks, W. J., Freeman, R. S., Gruda, M., Bravo, R., and Johnson, E. M. (1994). Altered gene expression during programmed cell death: Identification of c-*jun* as necessary for neuronal apoptosis. *J. Cell Biol.* **127,** 1717–1727.

Fan, H., Josic, D., Lim, Y. P., and Reutter, W. (1995). cDNA cloning and tissue-specific regulation of expression of rat calcium-binding protein 65/67. Identification as a homologue of annexin VI. *Eur. J. Biochem.* **230,** 741–751.

Fletcher, B. S., Lim, R. W., Varnum, B. C., Kujubu, D. A., Koski, R. A., and Herschman, H. R. (1991). Structure and expression of *TIS21,* a primary response gene induced by growth factors and tumor promoters. *J. Biol. Chem.* **266,** 14511–14518.

Fornace, A. J., and Mitchell, J. B. (1986). Induction of B2 RNA polymerase III transcription by heat shock: enrichment for heat shock induced sequences in rodent cells by hybridization subtraction. *Nucleic Acids Res.* **14,** 5793–5811.

Freeman, R. S., Estus, S., Horigome, K., and Johnson, E. M. (1993). Cell death genes in invertebrates and (maybe) vertebrates. *Curr. Opin. Neurobiol.* **3,** 25–31.

Freeman, R. S., Estus, S., and Johnson, E. M. (1994). Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of *cyclin D1* during programmed cell death. *Neuron* **12,** 343–355.

Gansheroff, Lisa J., Dollard, C., Tan, P., and Winston, F. (1995). The *Saccharomyces cerevisiae SPT7* gene encodes a very acidic protein important for transcription *in vivo. Genetics* **139,** 523–536.

Geisburg, J. V., Chen, J. L., and Ricciardi, R. P. (1995). Subregions of the adenovirus E1A transactivation domain target multiple components of the TFIID complex. *Mol. Cell. Biol.* **15,** 6283–6290.

Goodrich, J. A., and Tijan, R. (1994). TBP-TAF complexes: selectivity factors for eukaryotic transcription. *Curr. Opin. Cell Biol.* **6,** 403–409.

Grima, B., Lamouroux, A., Blanot, F., Biquet, E. E., and Mallet, J. (1985). Complete coding sequence of rat tyrosine hydroxylase mRNA. *Proc. Natl. Acad. Sci. USA* **82,** 617–621.

Hale, A. J., Smith, C. A., Sutherland, L. C., Stoneman, V. E., Longthorne, V. L., Culhane, A. C. and Williams, G. T. (1996). Apoptosis: molecular regulation of cell death. *Eur. J. Biochem.* **236,** 1–26.

Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M., and Rubin, L. L. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14,** 927–939.

Hamburger, V., and Levi-Montalcini, R. (1949). Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. Exp. Zool.* **111,** 457–468.

Hayashida, T., Sekiguchi, T., Noguchi, E., Sunamoto, H., Ohba, T., and Nishimoto, T. (1994). The CCG1/TAFII250 gene is mutated in thermosensitive G1 mutatns of the BHK21 cell line derived from golden hamster. *Gene* **141,** 267–270.

Haynes, S. R., Mozer, B. A., Bhatia-Dey, N., and Dawid, I. B. (1989). The *Drosophila fsh* locus, a maternal effect homeotic gene, encodes apparent membrane proteins. *Dev. Biol.* **134,** 246–257.

Haynes, S. R., Dollard, C., Winston, F., Beck, S., Trowsdale, J., and Dawid, I. B. (1992). The bromodomain: a conserved sequence found in human, *Drosophila* and yeast proteins. *Nucleic Acids Res.* **20,** 2603.

Hirschhorn, J. N., Brown, S. A., Clark, C. D., and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev.* **6,** 2288–2298.

Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992). Effects of *Rb* mutation in the mouse. *Nature* **359,** 295–300.

Konstantinova, I. M., Petukhova, O. A., Kulichkova, O. R., Turoverola, L. V., Volkova, I. V., Il'kaeva, O. R., Kozukharova, I. V., and Ermolaeva, Iu. B. (1995). A new class of RNP particles containing small RNA homologous to short dispersed DNA repetitive sequences. *Mol. Biol. (Mosk)* **29,** 761–771.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. *J. Neurosci.* **13,** 2739–2748.

Kramerov, D. A., Tillib, S. V., Lekakh, I. V., Ryskov, A. P., and Georgiev, G. P. (1985). Biosynthesis and cytoplasmic distribution

of small poly(A)-containing B2 RNA. *Biochim. Biophys. Acta* **824,** 85–98.

Krayev, A. S., Markusheva, T. V., Kramerov, D. A., Ryskov, A. P., Skryabin, K. G., Bayev, A. A., and Georgiev, G. P. (1982). Ubiquitous transposon-like repeats B1 and B2 of the mouse genome: B2 sequencing. *Nucleic Acids Res.* **10,** 7461–7475.

Kuwano, R., Usui, H., Maeda, T., Araki, K., Kurihara, T., Yamakuni, T., Ohtsuka, E., Isehara, M., and Takahashi, Y. (1986). Molecular cloning and nucleotide sequences of cDNA and genomic DNA for α and β subunits of S100 protein. *Taniguchi Symp. Brain Sci.* **9,** 243–255.

Lania, L., Pennuti, A., LaMantia, G., and Basilico, C. (1987). The transcription of B2 repeated sequences is regulated during transition from quiescent to proliferation state in cultured rodent cells. *FEBS Lett.* **219,** 400–404.

Lee, E. Y.-H. P., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and hematopoiesis. *Nature* **359,** 288–294.

Levi-Monatalcini, R., and Angeletti, P. U. (1966). Immunosympathectomy. *Pharmacol. Rev.* **18,** 619–628.

Lockshin, R. A. (1981). Cell death in metamorphosis. *In* ''Cell Death and Biology and Pathology'' (R. A. Lockshin and I. D. Bowen, Eds.), pp. 79–122. Chapman and Hall, New York.

Lygerou, Z., Conesa, C., Lesage, P., Swanson, R. N., Ruet, A., Carlson, M., Sentneac, A., and Seraphin, B. (1994). The yeast *BDF1* gene encodes a transcription factor involved in the expression of a broad class of genes including snRNAs. *Nucleic Acids Res.* **22,** 5332–5340.

Marcus, G. A., Silverman, N., Berger, S. L., Horiuchi, J., and Guarente, L. (1994). Functional similarity and physical association between GCN5 and ADA2: Putative transcriptional adaptors. *EMBO J.* **13,** 4807–4815.

Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G., and Johnson, E. M. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* **106,** 829–844.

Matsushime, H., Roussel, M. F., Asnmun, R. A., and Sherr, C. J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **65,** 701–713.

McKinnon, R. D., Danielson, P., Brow, A. D., Bloom, R. E., and Sutcliffe, G. R. (1987). Expression of small cytoplasmic transcripts of the rat identifier element *in vivo* and in cultured cells. *Mol. Cell. Biol.* **7,** 2148–2154.

Meikrantz, W., and Schlegel, R. (1995). Apoptosis and the cell cycle. *J. Cell. Biochem.* **58,** 160–174.

Mesner, P. W., Epting, C. L., Hegarty, J. L., and Green, S. H. (1995). A timetable of events during programmed cell death induced by trophic factor withdrawal from neuronal PC12 cells. *J. Neurosci.* **15,** 7357–7366.

Mills, J. C., Wang, S., Erecinska, M., and Pittman, R. N. (1995). Use of cultured neurons and neuronal cell lines to study morphological, biochemical and molecular changes occurring in cell death. *In* ''Methods in Cell Biology'' (L. M. Schwartz and B. A. Osborne, Eds.), Vol. 46, pp. 217–242. Academic Press, San Diego.

Naruse, I., and Keino, H. (1995). Apoptosis in the development of the CNS. *Prog. Neurobiol.* **47,** 135–155.

Oppenheim, R. W. (1991). Cell death during the developmnet of the nervous system. *Annu. Rev. Neurosci.* **14,** 453–501.

Oppenheim, R. W., Prevette, D., Tytell, M., and Homma, S. (1990). Naturally occurring and induced death in the chick embryo *in vivo* requires protein and RNA synthesis: evidence for a role of cell death genes. *Dev. Biol.* **138,** 104–113.

Orth, K., O'Rourke, K., Salvesen, G. S., and Dixit, V. M. (1996). Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases. *J. Biol. Chem.* **271,** 20977–20980.

Owens, G. P., Hahn, W. E., and Cohen, J. J. (1991). Identification of mRNAs associated with programmed cell death in immature thymocytes. *Mol. Cell. Biol.* **11,** 4177–4188.

Panoskaltsis-Mortari, A., and Bucy, R. P. (1992). *In situ* hybridization with digoxigenin-labeled RNA probes: Facts and artifacts. *BioTechniques* **18,** 300–307.

Park, D. S., Stefanus, L., Yan, C. Y. I., Farinelli, S. E., and Greene, L. A. (1996). Ordering of the cell death pathway. Differential effects of BCL2, an interleukin-1-converting enzyme family protease inhibitor, and other survival agents on JNK activation in serum/nerve growth factor-deprived PC12 cells. *J. Biol. Chem.* **271,** 21898–21905.

Patel, T., Gores, G. J., and Kaufmann, S. H. (1996). The role of proteases during apoptosis. *FASEB J.* **10,** 587–597.

Pattanakitsalkul, S., Zheng, J. H., Natsuume-Sake, S., Takahashi, M., and Nonaka, M. (1992). Aberrant splicing caused by insertion of the B2 sequence into an intron of the complement C4 gene is the basis for low C4 production in H-2k mice. *J. Biol. Chem.* **267,** 7814–7820.

Peterson, C. L., and Herskowitz, I. (1992). Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription. *Cell* **68,** 573–583.

Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993). Neurodegeneration induced by β amyloid peptides *in vitro*: The role of peptide assembly state. *J. Neurosci.* **13,** 1676–1687.

Pittman, R. N., Wang, S., DiBenedetto, A. J., and Mills, J. C. (1993). A system for characterizing cellular and molecular events in programmed neuronal cell death. *J. Neurosci.* **13,** 3669–3680.

Randazzo, F. M., Khavari, P., Crabtree, G., Tamkun, J., and Rossant, J. (1994). *brg1*: A putative murine homolog of the *Drosophila brahma* gene, a homeotic regulator. *Dev. Biol.* **161,** 229–242.

Rigby, P. W., LaThangue, N. B., Murphy, D., and Skene, B. I. (1985). The regulation of cellular transcription by Simian virus 40 large T-antigen. *Proc. R. Soc. Lond. B. Biol. Sci.* **226,** 15–23.

Rouault, J. P., Rimokh, R., Tessa, C., Paranhos, G., Ffrench, M., Duret, L., Garoccio, M., Germain, D., Samarut, J., and Magaud, J. P. (1992). *BTG1,* a member of a new class of antiproliferative genes. *EMBO J.* **11,** 1663–1670.

Rubin, L. L., Philpott, K. L., and Brooks, S. F. (1993). The cell cycle and cell death. *Curr. Biol.* **3,** 391–394.

Ruppert, S., Wang, E. H., and Tijan, R. (1993). Cloning and expression of human TAFII250: A TBP-associated factor implicated in cell-cycle regulation. *Nature* **362,** 175–179.

Russanova, V. R., Driscoll, C. T., and Howard, B. H. (1995). Adenovirus type 2 preferentially stimulates polymerase III transcription of Alu elements by relieving repression: A potential role for chromatin. *Mol. Cell. Biol.* **15,** 4282–4290.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). ''Molecular Cloning: A Laboratory Manual,'' 2ed. Cold Spring Harbor Laboratory Press, NY.

Sanders, E. J., and Wride, M. A. (1995). PCD in development. *Int. Rev. Cytol.* **163,** 105–173.

Saunders, J. W. (1966). Death in embryonic systems. *Science* **154,** 604–609.

Schwartz, L. M., Kosz, L., and Day, B. K. (1990). Gene activation is required for developmentally programmed cell death. *Proc. Natl. Acad. Sci. USA* **87,** 6594–6598.

Schwartz, L. M. (1992). Insect muscle as a model for programmed cell death. *J. Neurobiol.* **23,** 1312–1326.

Scott, S. A., and Davies, A. M. (1990). Inhibition of protein synthesis prevents cell death in sensory andprasympathetic neurons deprived of neurotrophic factor *in vitro*. *J. Neurobiol.* **21,** 630–638.

Shao, Z., Ruppert, S., and Robbins, P. D. (1995). The retinoblastoma-susceptibility gene product binds directly to the human TATA-binding protein-asscociated factor II250. *Proc. Natl. Acad. Sci. USA* **92,** 3115–3119.

Shearn, A. (1989). The *ash-1, ash-2* and *trithorax* genes of *Drosophila melanogaster* are functionally related. *Genetics* **121,** 517–525.

Smeyne, R., Vendrell, M., Hayward, M., Baker, S., Miao, G., Schilling, K., Robertson, L., Curran, T., and Morgan, J. (1993). Continuous *c-fos* expression precedes programmed cell death *in vivo*. *Nature* **363,** 166–169.

Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science* **267,** 1445–1449.

Sun, D., Sathyanarayana, U. G., Johnston, S. A., and Schwartz, L. M. (1996). A member of th e phylogenetically conserved CAD family of transcriptional regulators is dramatically up-regulated during programmed cell death of skeletal muscle in tobacco hawkworm Manduca sexta. *Dev. Biol.* **173,** 499–509.

Symonds, H., Krail, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. *Cell* **78,** 703–711.

Tamkun, J., Deuring, R., Scott, M., Kissenger, M., Pattatucci, A., Kaufman, T., and Kennison, J. (1992). *brahma*: A regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell* **68,** 561–572.

Tata, J. R. (1966). Requirement for RNA and protein synthesis for induced regression of tadpole tail in organ culture. *Dev. Biol.* **13,** 77–89.

Tso, J. Y., Sun, X. H., Kao, T. H., Reece, K. S., and Wu, R. (1985). Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: Genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.* **13,** 2485–2502.

Ucker, D. S. (1991). Death by suicide: One way to go in mammalian cellular development? *New Biol.* **3,** 103–109.

Wang, S., and Pittman, R. N. (1993). Altered protein binding to the octamer motif appears to be an early event in programmed neuronal cell death. *Proc. Natl. Acad. Sci. USA* **90,** 10385–10389.

Watson, J. B., and Sutcliffe, J. G. (1987). Primate brain-specific cytoplasmic transcript of the Alu repeat family. *Mol. Cell. Biol.* **7,** 3324–3327.

Weinzieri, R. O. J., Ruppert, S., Dynlacht, B. D., Tanese, N., and Tijan, R. (1993). Cloning and expression of *Drosophila* TAFII60 and human TAFII70 reveal conserved interactions with other subunits of TFIID. *EMBO J.* **12,** 5303–5309.

Welcher, A. W., Torres, A. R., and Ward, D. C. (1986). Selective enrichment of specific DNA, cDNA and RNA sequences using biotinylated probes, avidin and copper chelate agarose. *Nucleic Acids Res.* **14,** 10027–10043.

Wharton, K. A., Yedvobnick, B., Finnerty, V. G., and Artavanis-Tsakonas, S. (1985). opa: A novel family of transcribed repeats shared by the *Notch* locus and other developmentally regulated loci in *D. melanogaster*. *Cell* **40,** 55–62.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264,** 677–683.

White, R. J., Stott, D., and Rigby, P. W. J. (1989). Regulation of RNA polymerase III transcription in response to F9 embryonal carcinoma stem cell differentiation. *Cell* **59,** 1081–1092.

Wright, L. L., Cunninghamn, T. J., and Smolen, A. J. (1983). Developmental neuron death in the rat superior cervical sympathetic ganglion: Cell counts and ultrastructure. *J. Neurocytol.* **12,** 727–738.

Yuan, J., and Horvitz, H. (1990). The *Caenorhabditis elegans* genes *ced-3* and *ced-4* act cell autonomously to cause programmed cell death. *Dev. Biol.* **138,** 33–41.

Received for publication January 10, 1997
Accepted June 6, 1997